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Abstract: Aquaculture industry exposes fish to acute stress events, such as high stocking density, and a link between stress and higher susceptibility to diseases has been concluded. Several studies have demonstrated increased stress tolerance of fish treated with probiotics, but the mechanisms involved have not been elucidated. Shewanella putrefaciens Pdp11 is a strain isolated from healthy gilthead seabream (Sparus aurata L.) and it is considered as probiotics. The aim of this study was to evaluate the effect of the dietary administration of this probiotics on the stress tolerance of Solea senegalensis specimens farmed under high stocking density (PHD) compared to a group fed a commercial diet and farmed under the same conditions (CHD). In addition, during the experiment, a natural infectious outbreak due to Vibrio species affected fish farmed under crowding conditions. Changes in the microbiota and histology of intestine and in the transcription of immune response genes were evaluated at 19 and 30 days of the experiment. Mortality was observed after 9 days of the beginning of the experiment in CHD and PHD groups, it being higher in the CHD group. Fish farmed under crowding stress showed reduced expression of genes at 19 day probiotic feeding. On the contrary, a significant increase in immune related gene expression was detected in CHD fish at 30 day, whereas the gene expression in fish from PHD group was very similar to that showed in specimens fed and farmed with the conventional conditions. In addition, the dietary administration of S. putrefaciens Pdp11 produced an important modulation of the intestinal microbiota, which was significantly correlated with the high number of goblet cells detected in fish fed the probiotic diet.

***Highlights**

- *S. putrefaciens* Pdp11 enhances tolerance to crowding stress in *Solea senegalensis*
- Dietary administration of *S. putrefaciens* Pdp11 modulates immune gene transcription
- Some intestinal changes are related to microbiota modulation by *S. putrefaciens* Pdp11

The treatment with the probiotic *Shewanella putrefaciens* Pdp11 of specimens of *Solea senegalensis* exposed to high stocking densities to enhance their resistance to disease

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Abstract

Aquaculture industry exposes fish to acute stress events, such as high stocking density, and a link between stress and higher susceptibility to diseases has been concluded. Several studies have demonstrated increased stress tolerance of fish treated with probiotics, but the mechanisms involved have not been elucidated. *Shewanella putrefaciens* Pdp11 is a strain isolated from healthy gilthead seabream (*Sparus aurata* L.) and it is considered as probiotics. The aim of this study was to evaluate the effect of the dietary administration of this probiotics on the stress tolerance of *Solea senegalensis* specimens farmed under high stocking density (PHD) compared to a group fed a commercial diet and farmed under the same conditions (CHD). In addition, during the

experiment, a natural infectious outbreak due to *Vibrio* species affected fish farmed under crowding conditions. Changes in the microbiota and histology of intestine and in the transcription of immune response genes were evaluated at 19 and 30 days of the experiment. Mortality was observed after 9 days of the beginning of the experiment in CHD and PHD groups, it being higher in the CHD group. Fish farmed under crowding stress showed reduced expression of genes at 19 day probiotic feeding. On the contrary, a significant increase in immune related gene expression was detected in CHD fish at 30 day, whereas the gene expression in fish from PHD group was very similar to that showed in specimens fed and farmed with the conventional conditions. In addition, the dietary administration of *S. putrefaciens* Pdp11 produced an important modulation of the intestinal microbiota, which was significantly correlated with the high number of goblet cells detected in fish fed the probiotic diet.

Introduction

Aquaculture is a highly competitive growth industry which, in the search for greater economic yields and increased production, exposes fish to acute stress events, such as the increase of the stocking density. Stress is a situation in which organisms are subjected to challenges that may result in a danger to their integrity [1]. Fish diseases constitute major limiting factors in aquaculture [2] and there is a strong link between stress and susceptibility to such diseases [3] based on close interactions between the stress-immune system [1]. It has been shown that in most cases, diseases appear when animals have previously been exposed to a stress situation [1].

Contradictory results regarding the effects of high stocking densities on fish performance have been reported. Thus, whereas low growth has been observed for

farmed halibut (*Paralichthys californicus*) [4] and Dover sole (*Solea solea*) [5] exposed to high stocking densities, a null effect on the growth of Senegalese sole (*Solea senegalensis*) has been reported [6, 7]. However, there is increasing evidence that social stressors such as crowding may exert immune suppression [1] and can significantly affect mucosal surface components of the gastrointestinal tract, the intestinal microbiota [8] and have an important effect on fish nutrition and host immunity [9, 10]. Mucosal surfaces particularly in fish represent a potential route of entry for some pathogens [11, 12], but although the relationship between the status of the intestinal microbiota with disease incidence has been previously suggested [13], there is scarce information about the modulation of the intestinal microbiota of fish reared at different stocking densities [14].

Several studies have demonstrated an improvement to stress tolerance in fish treated with probiotics [15, 16, 17] and the potential role of probiotic microorganisms in overcrowded stress situations has been evaluated in different animals including fish. In this context, [18] reported positive effects on immunological response in nursery pigs stressed by reduced spaces and challenged with enterotoxigenic *Escherichia coli* (ETEC) $K88^{+}$. In fish, *S. putrefaciens* Pdp11, a probiotic strain isolated by us from farmed gilthead seabream (*Sparus aurata*, L.) and showing several probiotic characteristics [19], promoted growth and improved stress tolerance of gilthead seabream specimens cultured under high stocking density [20]. On the other hand, [21] evaluated the effects of a probiotic strain of *Lactobacillus rhamnosus* on the crowding stress response of specimens of Nile tilapia (*Oreochromis niloticus*), reporting that the probiotics increased energy availability for metabolic support of the crowding stress response and improved the stress coping capacity of fish. The probiotic *Shewanella putrefaciens* Pdp11 has also induced improved stress tolerance of gilthead seabream

specimens exposed to high stocking densities [20]. There is little information concerning the responses of *Solea senegalensis*, a flatfish in great demand in Southern European aquaculture markets [6], farmed under high stocking densities [7], and there is null information concerning the effect of this stressor on the intestine and the potential role played by probiotic microorganisms.

Fish have several protective mechanisms to prevent microbial entry through the gastrointestinal (GI) tract, including the production of mucus by goblet cells [22]. Disruption of the ecological equilibrium in the intestinal microbiota of the GI tract produces an increase in the permeability and alterations of the immune defences leading to a loss of the barrier function [22], and the possibility of bacterial translocation [23].

The aim of this study was to elucidate changes produced in the intestinal tract and the gene expression related to the immunological response of Senegalese sole specimens under high stocking density and receiving the dietary administration of the probiotic *S. putrefaciens* Pdp11, and the correlation that these factors could exert on their resistance to the disease.

Materials and Methods

Microorganisms

Shewanella putrefaciens Pdp11 isolated from healthy gilthead seabream skin was shown to be non-virulent for *Solea senegalensis* (Kaup) and *Sparus aurata* (L.), and it is capable of counteracting adhesion of pathogens to fish surfaces as well as enhancing immune activities of gilthead seabream and Senegalese sole leucocytes [24, 25, 26]. In addition, *Shewanella putrefaciens* Pdp11 increases growth rates of juvenile

soles [27] and resistance against pathogens such as *V. harveyi* and *Photobacterium damselae* subsp *piscicida* [24, 25, 26, 28]. For these reasons, it is considered a potential fish probiotics to be tested in farmed Senegalese sole and gilthead sea bream [19].

Shewanella putrefaciens Pdp11 cells were cultured in tryptone soya broth (Oxoid Ltd., Basingstoke, UK) supplemented with 15 g l^{-1} NaCl (TSBs) for 18 h at 22 ºC, with continuous shaking, and appropriate dilutions of the cultures were spread onto tryptone soya agar (Oxoid Ltd, Hampshire, UK) plates supplemented with 1.5% NaCl (TSAs). Bacterial suspensions were prepared by cell scraping from the plates and washing in sterile phosphate-buffered saline (PBS, pH 7.4). The number of culturable cells was determined by plate count on TSAs, and adjusted to 10^{11} CFU ml^{-1}.

Fish treatments and natural infection

One hundred and forty healthy farmed Senegalese sole specimens (14.6 ± 0.7 g mean body weight) from the stocks of the Spanish Oceanographic Institute of Santander (IEO, Spain) were randomly distributed in 150-L seawater tanks connected to an open circulating system (35.4 g l^{-1} salinity, 16.6 °C±0.5 °C), and with a renovation rate of 300% day^{-1}. They were acclimated for 2 weeks prior to the experimental period, and ten fish were sampled for bacteriology to check the health status of the stock. The specimens were dissected and samples of spleen, liver and kidney were cultured on TSAs at 22 ºC for 48 h to detect the potential presence of microorganisms. Fish were fed eight times a day [29] with a commercial pellet diet Gemma 1.8 Diamont (18% total lipids and 55% crude protein, SKRETTING, Burgos, Spain). The initial fish weight was used to adjust the daily ration of feed according to the normal pattern in this species (20 g kg^{-1}).

After the acclimation period, fish were randomly distributed in eight tanks. The diet previously described was supplied to four tanks. Fish specimens were farmed under normal stocking density (7 kg m^{-2}) in two tanks of each diet group (CND groups), whereas the stocking density in the other two tanks was higher (30 kg m^{-2}) (CHD groups). The rest of the tanks received the commercial feed supplemented with *Shewanella putrefaciens* Pdp11 bacterial cells. Ten milliliters of the bacterial suspension previously prepared were added to 90 ml of 0,5% (w/v) sodium alginate suspension (Sigma, Sigma-Aldrich, St Louis, MO, USA). The suspension was sprayed onto the feed following the methodology described by [30], to obtain a dose of 10^9 CFU g^{-1} feed which produces benefits in *S. senegalensis* [27].

Disease symptoms consisting in the presence of wounds on the skin and mortality started to be observed 9 days after the beginning of the experiment in fish groups maintained under high stocking density. Fish were not treated and a monitoring of the mortalities was carried out.

Sampling and processing of diseased fish

Moribund fish exhibiting hemorrhagic lesions on both body surfaces and pale livers were sampled for bacteriological analysis at days 9, 19 and 30. Samples from ulcers, head kidney and liver were cultured on TSAs and in TSBS. All inoculated media were incubated at 22ºC for 24 h, and the different colony types grown on the plates were picked and identified by the amplification and sequencing of a fragment of 16S rDNA. Briefly, this fragment was amplified using the universal primers SD-Bact-0008-a-S20 (5′ AGA GTT TGA TCC TGG CTC AG 3′) and SD-Bact-1492-a-A-19 (5′ GGT TAC CTT GTT ACG ACT T) [31]. Polymerase chain reactions were carried out in a 50 μL reaction mixture that included 5 pmol of each primer, 200 μMdNTPs, 1×PCR buffer,

2 mM$MgCl_2$, 1 U BIOTAQ™ DNA Polymerase (Bioline, London, UK) and 1 μL of a boiled colony suspension. The PCR profile was as follows: 2 min at 95 °C and 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 1.3 min at 72 °C and a final step of 5 min at 72 °C. Polymerase chain reaction products were electrophoresed on a 1% agarose gel and visualized via ultraviolet transillumination. Following the PCR reaction, unconsumed dNTPs and primers were removed using the High Pure PCR Product Purification kit (Roche, Mannheim, Germany). The PCR products were sent to Macrogen Inc. (Korea) for their sequencing, using SDBact-0008-a-S20 and SD-Bact-1492-a-A-19 as sequencing primers. The sequences were aligned to the closest relative in the GenBank database using BLAST.

Analysis of the intestinal microbiota

The individual intestines of three specimens from each tank (six per treatment) were sampled at days 0, 19 and 30. Intestinal contents were collected with 1 ml PBS, pH 7.2, and a 1 mL aliquot was centrifuged at 1000 × g for 5 min. Total DNA was extracted from each sample as described by [32]. Pure culture of the probiotic strain *S. putrefaciens* Pdp11 was grown to exponential phase in TSBs, and then centrifuged at 2500 × g for 15 min. Pellets were washed with PBS and used for DNA extraction following Fast DNA Spin kit (Qbiogene, CA. USA) instructions. DNA was amplified using the 16S rDNA bacterial domain-specific primers 968-GC-F (5'GA-ACGCGAAGAACCTTAC-3') and 1401-R (5'CGGTGTGTACAAGACCC-3'). Primer 968-CG-F carries a 35 bp GC clamp. PCR mixtures and conditions to perform PCR were those previously described by [30]. The amplicons obtained were separated by Denaturing Gradient Gel Electrophoresis (DGGE) according to the specifications of

[33] using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA). The gels were subsequently stained with $AgNO_3$ [34].

DGGE banding patterns were analyzed using FPQuest Software version 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). A matrix of similarities for the densitometric curves of the band patterns was calculated using the Bray-Curtis index. Clustering of DGGE patterns was achieved by construction of dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA). Structural diversity of the microbial community was determined by an analysis of the DGGE-patterns. Species richness (R) was calculated based on the total number of bands, and Shannon index (H′) was also calculated following the function: $H' = -\Sigma$ Pi log Pi, where Pi is defined as (ni/N), ni is the peak surface of each band, and N is the sum of the peak surfaces of all bands.

Cloning of the PCR amplified products

DNA was extracted and PCR performed with a Taq DNA polymerase kit from Life Technologies. The forward primer S-D-Bact-0008-aS-20 (5’ AGA GTT TGA TCC TGG CTC AG 3’) [35], which targets the domain Bacteria, and the reverse primer S-*-Univ-1492-b-A-21 (59ACG GCT ACC TTG TTA CGA CTT 3’) [36] which amplifies the bacterial 16S rDNA were used. Amplification was carried out as described by [37]. Reaction tubes contained 100 ng total DNA, 1.25 U Taq DNA polymerase (from Life Technologies), buffer, 2.5 mM $MgCl_2$, 200 mM each deoxyribonucleotide triphosphate, and 10 µM each primer in a final volume of 50 uL. Initial DNA denaturation and enzyme activation steps were performed at 94 °C for 10 min in a Eppendorf thermocycler, followed by 30 cycles denaturation at 92°C for 1 min, annealing at 48 °C for 1 min, and elongation at 72 °C for 1 min 30 s, followed by final elongation at 72 °C

for 5 min. PCR products were purified and concentrated with High Pure Spin Kit PCR purification kit (Roche) according to the manufacturer instructions. Purified PCR product was cloned into pGEM-T vector (Promega,Madison, WI, USA). Ligation was performed at 4 ºC overnight followed by transformation into competent *E. coli* JM109. One hundred colonies of ampicillin-resistant transformants from each diet and day sampled were transferred with a sterile toothpick to 100 µL TE buffer and boiled for 10 min at 95ºC. Immediately, PCR was performed with pGEMT- specific primers T7 (5'-AAT ACG ACT CAC TAT AGG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG-3'). Plasmids containing an insert were used to amplify V6-V8 region of 16S rDNA. The amplicons were compared with the bands of DGGE patterns and used for sequence analysis (Macrogen Korea sequencing). They were compared with the sequences from the National Center for Biotechnology Information (NCBI) using the BLAST sequence algorithm [38]. Database sequences showing the highest correlation were used to infer identity.

Statistical analysis

Bray-Curtis similarity index was calculated based on DGGE densitometric curves and used for cluster analysis (UPGMA method) and Non Metric Multidimensional (NMDS) plots. An analysis of similarities (ANOSIM) to test for significant differences between control and treatment groups was carried out using PAST version 2.17 software [39]. The contribution of clones identified from cloning experiments to similarity percentages (SIMPER) between control and treatment groups was determined using PAST software. The significance of differences of the Shannon index (H'), and

species richness (R) values was determined after performing analysis of variance (ANOVA) with XLSTAT 2013 software (Addinsoft, Madrid, Spain).

Kruskal–Wallis test was used to test the significance of the differences in species richness (R) and species diversity (H′) using the program STATGRAPHICS Plus 5.0 (Statgraphics Corporation, Rockville, MD, USA). Principal Components Analysis (PCA) was applied to correlate variables determined in the liver of fish fed assayed diets and microbial species detected in the intestinal microbiota using XLSTAT 2012 software (Addinsoft, Madrid, Spain) and orthogonal transformation (Varimax).

Absolute transcript quantification by real-time RT-PCR

The livers of at least five specimens per treatment at days 0, 19 and 30 were dissected, individually frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted from individual livers, using TRIZOL reagent (Invitrogen, Spain) according to manufacturer instructions. Genomic DNA contamination was avoided by subsequent cleanup with RNeasy reagents (Qiagen Inc., CA, USA). RNA quality was checked using an Agilent 2100 bioanalyzer and quantification was done spectrophotometrically. cDNA was generated from 2 µg of total RNA from each sample, using the M-MLV reverse transcriptase (Life Technologies SA, Spain) and random hexamers (Invitrogen, Spain). For quantifications on pooled samples, one RNA pool was prepared for each experimental condition by combining equal amounts of the individual RNA samples before retrotranscription.

Absolute real-time qRT-PCR was performed as described by [40]. Primers directed against the selected genes were as described previously in [40] (NCCRP1, C3, C7, and HP) and [41] (HAMP1, TNFAIP9, and NARS) or designed with the Oligo 6.1 software (Molecular Biology Insights), as detailed in [42] (LYZ, HSP90AB and

HSP70) (Supplementary Table 1). To obtain high specificity and performance, primers were required to have high Tm (≥ 80 ºC), optimal 3-ΔG (≥ −3 kcal/mol) value, and to be hairpin and duplex free. All primer pairs produced amplicons of the predicted size.

Real-time PCR reactions, both on pooled or individual samples, were performed in quadruplicate using 50 ng of cDNA template, 0.3 µM of each primer, 3mM $MgCl_2$, 250 µM of each dNTP, 0.75 units of Platinum Taq DNA polymerase, and 1:100,000 SYBR Green I dye (Roche, Spain) in a volume of 25 µl. Reactions were analyzed on an *CFX96 Real-Time PCR System* (BioRad, Life Technologies SA, Spain). Cycling conditions were as follows: 2 min at 95 ◦C for the Platinum Taq activation and 40 cycles for the melting (15 s, 95 ◦C) and annealing/extension (30 s, 70 ◦C) steps. Following denaturation at 95 °C for 15 s, and cooling to 60 °C for 30 s, a melt curve (Tm) was generated by heating from 60 °C to 95 °C with 0.5 °C increments, 10 s dwell time and fluorescence read at each temperature. The specificity of the reactions was confirmed by the Tm curves, which were consistently specific for the amplified products. No primer dimers were detected and all PCR products were further verified by nucleotide sequencing. Replicate PCR reactions generated highly reproducible results with S.E.M. <10% of the mean (<1% for threshold cycle). The reliability of an absolute quantification depends on identical amplification efficiencies for both the target and the calibrator. All primers amplified with the same optimal PCR efficiency (~100%) in the range of 20 to $2x10^5$ pg of total RNA input with high linearity (r≥0.98) (Supplementary Table 1) [40, 41]. The number of transcript molecules was calculated from the linear regression of the absolute calibration curve ($y = -3.326x + 39.693$; 100% efficiency), as described previously by [43, 44].

Light microscopy study

For light microscopy (LM) study, samples of anterior intestine were fixed in fresh 10% neutral buffered formalin at room temperature for 24 h. After serial dehydration steps in alcohol, samples were embedded in hydrophilic resin JB-4 according to routine procedures. Sections were cut at 3 μm (Microm), mounted on silane-covered (TESPA, 3-aminopro- pyl-triethoxy-silane; Sigma, Spain) glass slides, and stained with haematoxylin and eosin (HE) for histological analysis. Sections were also stained for neutral and acid mucins using Alcian blue (AB) and periodic acid-Schiff (PAS) [45].

Histological screening and intestinal morphometry

The effects of dietary treatments were monitored by LM in terms of loss of epithelial integrity, presence of cell debris in the lumen, villus height, presence of goblet cells, intraepithelial and lamina propia leucocytes, villus and wall intestinal areas, microvilli disorganization / disruption, and oedema. Computerised morphometric measurements were made of the following: (1) villus height (Vh, μm, measured from the tip to the base of villus) and intestinal diameter (Id, μm), (2) number of goblet cells per area of epithelium layer (total, PAS+ and PAS+AB+), (3) intra-epithelial leucocytes (IELs) and lamina propria leucocytes (LPLs), and (4) lumen, villus, and wall areas. The Vh was corrected to Id to take into account the variations which may occur due to increased intestinal diameter. For this, ratio Id / Vh was calculated for all the samples. These measures were made using MIP 4.5 image analysis software (Consulting Image Digital, Spain) and NewCAST software (Visiopharm, Hoersholm, Denmark). Images were obtained with Leica 6000B microscopy equipped with a Leica DFC280 camera and Leica Application Suite V 2.5.0 R1 acquisition software (Leica Microsystems SLV, Spain). Results were expressed as mean values with their standard errors.

The signs observed were assessed against arbitrary units in order to obtain numerical data that would enable us to statistically analyze the observed differences with respect to control samples. Each change received an arbitrary score from 0 to 3: 0—none/normal, 1—mild, 2—moderate, and 3—marked. Total anterior intestine scores were classified as: 0–1, normal; 1–3, moderate pathological changes; >3–6 severe pathological change [46, 47].

Results

Bacterial infection

Fish exposed to high stock density conditions showed darkness and wounds on the skin (Supplementary Figure 1) one week after stocking and starting feeding treatment. The intensity of the symptoms was higher in CHD fish compared to PHD specimens. Mortality was detected in the CHD group from day 9 of the experiment, reaching 40% after 30 days, whereas mortality in the PHD group was below 5% (Figure 1) and it stopped at 11 day of the experiment. Only two different types of colonies on TSAs identified as *V. harveyi* (HM584117) and *V. parahaemolyticus* (JK134442 and KC210810) were isolated by bacteriological analysis of internal organs, whereas they were the predominant colony types from wounds sampled.

Effect of stocking density on intestinal microbiota

Cluster analysis was carried out using UPGMA methods based on the Bray-Curtis similarity of densitometric DGGE bands obtained at 19 and 30 days (Figure 2). Clusters grouping specimens based on the diet and sampling day were observed both in fish

exposed to normal and high stocking densities. The percentage of similarity among the specimens of both diet groups was very high (>80%). Similar results were visualized in NMDS plots (Figure 3) showing significant differences ($p<0.01$) in the intestinal microbiota among groups receiving the control and the probiotic diets at different days and exposed to different stocking densities. Ecological parameters summarized in Table 1 indicate that high stocking densities significantly ($p<0.05$) reduced the richness (R) and the Shannon's diversity index (H') in the DGGE patterns, this reduction being more marked when the specimens received the probiotic diet for 30 days.

One hundred clones were sequenced from each experimental group assayed and the closest relatives as determined by comparative 16S rDNA sequence analysis are summarized in Table 2. γ-Proteobacteria and Mollicutes were present in fish intestinal microbiota of all groups (Figure 4). Actinobacteria were not detected in the intestinal microbiota of specimens fed the probiotic diet, regardless of the farming stocking density assayed. However, γ-Proteobacteria was the most frequently sequenced bacterial group in specimens from all groups assayed, ranging from 41% to 73% (Figure 4). On the other hand, all clones related to Firmicutes phylum and sequenced from the GI tract of specimens from CND and PND groups were related to *Candidatus* Arthromitus (Clase *Clostridia*), whereas clones related to *Lactobacillus* genus (Clase *Bacilli*) were detected in the intestinal microbiota of fish exposed to high stocking density (Figure 5). Important changes in γ-Proteobacteria Clase were observed in the intestinal microbiota of fish based on the farming stocking density (Figure 5). In this context, clones related to *Pseudomonas* genus were only sequenced from the GI tract of fish from CHD and PHD groups. On the other hand, clones related to *Photobacterium damselae* subsp *piscicida* were not sequenced when fish were exposed to high stocking density and in those specimens from PND group after 30 days receiving the probiotic diet.

Clones related to *Vibrio* genus were the most frequently sequenced microorganisms from the intestinal microbiota of the majority of fish. Those related to Harveyi clade, such as *V. parahaemolyticus*, were the most frequently sequenced from the GI tract of specimens from the CND and CHD groups, ranging from 10% to 25% (Figure 5). However, the intestinal microbiota of CHD fish showed higher species diversity of this clade and clones related to *V. campbellii* and *V. harveyi* were only detected in specimens from this group. On the contrary, *V. campbellii* and *V. harveyi* were not detected in the GI tract of fish fed the probiotic diet regardless of the stocking density assayed, whereas clones related to *V. ichthyoenteri* and *V. scophthalmi* (3%) were only detected in the intestinal microbiota of these specimens. Due to the fact that mortality was detected in specimens from CHD and PHD groups, similarity percentage analysis (SIMPER) values to determine the contribution of each bacterial group to the observed dissimilarity between the CHD and PHD groups are summarized in Table 3. Differences at 19 days were attributed to γ-Proteobacteria (49.65%) and Firmicutes (25.52%), whereas differences at 30 days were due to γ-Proteobacteria (23%), and unidentified bacteria (22.92%).

Effect of stocking density on immune gene expression

Values of quantifications firstly made on pooled RNA from specimens of each experimental group, sampled at 19 and 30 days after the beginning of the experiment are summarized in Table 4. Given that it is not possible to perform a statistical analysis with pooled samples, we arbitrarily established changes involving variations ≥ 2.0-fold as being different. Following this criterion, at 19 days C7, HP, HSP70, G-LYS, and NARS showed a decrease in their mRNA copy number when fish were exposed to high

stocking density, regardless of treatment with or without the probiotic (CHD and PHD in comparison to CND and PND). HSP70 and G-LYS exhibited a similar behaviour at 30 days. In contrast, control fish collected at 30 days from the high density group (CHD) exhibited higher hepatic levels of C7, HAMP1, HP, NARS, NCCRP1 and TNFAI9 transcripts, in comparison with fish farmed under a normal stocking density (CND). Notably, in fish fed the probiotic diet (PHD) the amounts of these transcripts declined, recovering similar levels to those obtained in fish farmed under a normal stocking density (CND and PND).

In order to validate accurately the up- and down-regulation of the transcripts and determine the effect of inter-individual variability, absolute real-time PCR quantifications at individual level was carried out. Data at individual level (Figure 6) confirmed the results obtained in samples from mixtures of individuals.

Effect of stocking density on the histology of the intestinal tract

Histological changes in the anterior intestine of fish from the different experimental groups were assessed by light microscopy (LM) and morphometric measurements are summarized in Table 5. Statistically significant decreases in the villus height and diameter of the intestine were observed in fish from high stocking density groups (both, CHD and PHD groups) sampling being taken at 19 and 30 days. Similarly, intestine villus height was affected by high stocking density condition this decrease being more pronounced in the CHD group. The inclusion of the probiotic in fish diet and/or stocking fish at high density (PND, CHD and PHD groups) resulted in a statistically significant decrease of the diameter of the intestine, in comparison with the

values found in the intestine of fish fed with the control diet and farmed under a normal stocking density (CND group).

The total number of goblet cells was similar in all groups at 19 days, but they were significantly higher in fish farmed under a high stocking density, especially in the PHD group at 30 days (Table 5). However, the types of goblet cells were affected in a different manner. No statistically significant differences were observed among fish from the different experimental groups after 19 days treatment regarding the number of PAS+ cells. However, after 30 days treatment, a statistically significant increase was observed in such cells in fish from the PHD group. Furthermore, a statistically significant increase was observed in the number of PAS+AB+ cells in the intestine of fish from the PHD group, and a drastic decrease in fish from the PND group, in comparison the group of fish fed the control diet and farmed under a normal stocking density (CND groups).

In summary, the analysis of the samples from gut tracts of the fish under high stocking densities and receiving a probiotic diet showed significant decreases in the intestine villus height and diameter and significant increases in the number of goblet cells.

Principal Components Analysis (PCA)

The results of PCA evaluating the relationship between the status of the intestinal microbiota, gene expression, epithelial barrier and mortality rates at 30 days are shown in Figure 7. Two principal components account for about 70% of the total data variability. PCA showed a clear differentiation of microbial clones into two groups along the first PC axis F1 (Figure 7A). It is possible to observe that the total number of goblet cells (PAS) is correlated with the presence of microbial clones only present in the

intestinal microbiota of fish exposed to high stocking density and fed the probiotic diet. Fish exposed to high stocking density and fed the control and probiotic diets (CHD and PHD groups, respectively) were differentiated in two clear groups: specimens from the CHD and PHD groups scoring positive and negative on F1 axis, respectively (Figure 7B).

High stocking density accounted for almost 30% total variance of component F2, and fish kept under high stocking were separated along F2 axis from ND fish. However, the distance in F2 axis between HD and ND stocked fish was lower for fish fed the probiotic diet, indicating attenuation of the influence of stress density.

Discussion

Stress increases susceptibility to infectious diseases in fish [48, 49]. In this context whereas fish exposed to normal stocking densities did not show any symptoms of disease throughout the experimental trial, specimens of high density groups fed both diets (CHD and PHD) suffered an infectious outbreak with typical symptoms of vibriosis [50]. Strains of *V. harveyi* and *V. parahaemolyticus* were isolated and identified from the internal organs of diseased fish. These two species are included in the Harveyi clade (or *Vibrio* core group) proposed by [51], that includes *V. harveyi*, *V. campbellii*, *V. rotiferianus*, *V. parahemolyticus*, *V. alginolyticus*, *V. natriegens* and *V. mytili*. Some of them include important harmful strains for aquatic organisms [52, 53, 54] and *V. harveyi* and *V. parahaemolyticus* are pathogenic species for *Solea senegalensis* [55].

The ability showed by *S. putrefaciens* Pdp11 to modulate the intestinal microbiota of *Solea senegalensis* has been previously reported [28, 30], and this has been

corroborated in this study. In this context, the administration of the probiotic diet reduced the presence of clones related to Actinobacteria and *Photobacterium damselae* subsp *piscicida*, and those included in the Harveyi clade. On the contrary, 30 days after the beginning of the experiments and coinciding with the mortality peak observed in the CHD group, high percentages of clones related to *V. parahaemolyticus* and *V. harveyi* were present in these fish intestinal microbiota. In contrast, fish from the PHD group showed lower diversity rates and a lower number of clones related to Harveyi clade than fish from CHD group, and *V. harveyi* was not detected in any fish. In addition, fish from PHD group contained clones related to *V. ichthyoenteri* and *V. scophthalmi* which have been reported as a siderophore-producing bacteria and a potential probiotic candidate, respectively [56]. The ability shown by some bacteria to produce siderophores has been proposed as a potential probiotic mechanism against the pathogens [57, 58, 59]. Although the effect of probiotic diet on fish intestinal microbiota was clearly observed, bands related to *S. putrefaciens* Pdp11 were not detected from the intestines of fish fed this diet. The absence of these bands may not exclude the presence of the probiotic cells, since they could be under the detection limit of the DGGE technique which has an abundance limit of 1% [60]. In addition, the ability to colonize Senegalese sole intestine by *S. putrefaciens* Pdp11 has been reported when fish have received the same probiotic for longer periods [61, 62].

The relationship between stress, immunosupression and disease incidence has been suggested [63]. In this way, specimens included in the CHD and PHD groups showed significant immunosupression at 19 days, given that these animals exhibited decreased levels of several transcripts coding for proteins that have been previously described as relevant in the innate immune system of *S. senegalensis* [40, 41], notably as a part of the acute phase response (C7, HP, and NARS) or playing a direct role in the

defence against pathogenic bacteria (G-LYZ). This result is in agreement with those reported by [7] where a marked reduction in mRNA levels of G-LYS and HSP70 (a molecular chaperon) was observed and associated with elevated plasma levels of cortisol detected in *S. senegalensis* specimens farmed under crowding conditions. Our results are also in consonance with a decrease in the plasmatic lysozyme and alternative complement pathway described in a recent study in this fish species [64]. Overall, our data corroborate the evidence that social stressors may exert an immunological depression as has been proposed by [1]. However, at 30 days fish from the CHD group showed up-regulation of the majority of the immune related genes studied. These include the aforementioned C7, HP and NARS, as well as HAMP1, TNFAIP9, and NCCRP1. HAMP1 codes for an antimicrobial peptide that plays an important role in defence against invading pathogens, TNFAIP9 for a protective anti-inflammatory factor and NCCRP1 for a non-specific cytotoxic cell receptor protein involved in the antigen-binding, signaling and transcriptional regulation processes. All these genes have shown up-regulation of their transcriptional expression in specimens of *S. senegalensis* inoculated with LPS [40, 41]. Hence, expression data clearly indicate that fish from the CHD group presented a microbial infection. Nevertheless, the levels of these transcripts in specimens exposed to high stocking density but fed the probiotic diet (PHD) were similar to those exhibited by fish farmed under normal densities for most genes assayed. It could indicate that the administration of *S. putrefaciens* Pdp11 assists recovery of the specimens after infection.

The higher intensity of symptoms showed by fish from CHD groups together with the fact that *V. harveyi* (HM584117) and *V. parahaemolyticus* (KC210810 and JK134442) only were isolated from skin wounds and internal organs of specimens of the CHD group may indicate that the potential route of pathogens entry could be the

wounds on the skin and from this site, dissemination could occur through the blood. *V. parahaemolyticus* and *V. harveyi* were described as aetiological agents of outbreaks affecting specimens of *Solea senegalensis* and described as able to produce ulcers on the skin and moderate mortality [55]. However, the gastrointestinal tract is also an important portal entry of pathogens [65, 66]. Thus, *V. harveyi* has been isolated from marine fish affected by gastroenteritis syndrome [67] while *V. parahemolyticus* is considered an important intestinal pathogen [68, 69]. In this context, clones related to strains isolated from skin wounds and internal organs were detected in the intestinal microbiota of fish from CHD group, and for this reason, this route could be considered as a potential portal entry of *V. harveyi* and *V. parahaemolytcus*. It is well known that stress induces alterations of the intestinal barrier, such as damage to fish enterocytes and changes in the intestinal microbiota [8, 70, 71], these findings being able to facilitate bacterial translocation. Bacterial translocation has been reported for indigenous and pathogenic bacteria, including some *Vibrio* species (reviewed by [22]). Changes in the intestinal barrier such as villus height, intestine diameter, number of goblet cells and intestinal microbiota of CHD group fish have been observed and it could facilitate translocation of *V. parahaemolyticus* and *V. harveyi* across the intestinal tissues. In addition to clones detected in intestines and related to *V. parahaemolyticus and V. harveyi*, other clones such as *Pseudomonas putida*, *Vibrio campbellii* and *Candidatus* Arthromitus were also detected, and they have been described as pathogens for fish. *P. putida* is an opportunistic pathogen responsible for bacteriemia in compromised hosts [72, 73], but it is not a common pathogen in aquaculture [74]. Some *V. campbellii* strains have been characterized as very pathogenic for aquatic organisms such as shrimps [75], whereas others were not pathogenic [76]. On the other hand, *Candidatus* Arthromitus is a not yet culturable bacteria, and it is responsible for rainbow trout

gastroenteritis syndrome [77]. The presence of these clones in the intestine but not in internal organs seems to corroborate the possibility that the portal entry of the pathogens in this work are the ulcers on skin. However, it is suggested that opportunistic fish pathogens, such as *V. parahaemolyticus* and *V. harveyi*, could remain in the mucus layer on the GI tract by adhering to mucus proteins [78] and in this way pass through the mucus layer and adhere to intestinal cells. In addition, [78] have reported the ability of *V. harveyi* cells to adhere to epithelial cells in the intestinal tract of red sea bream (*Pagrus major*) and [79] concluded that *V. parahaemolyticus* caused transepithelial resistance disruption of the M cell-like Caco-2/Raji B co-culture model system and translocated in high numbers across it. Obviously, more experiments are needed to demonstrate the potential translocation of pathogens such as *V. parahaemolyticus* and *V. harveyi* from the fish intestine to extraintestinal sites.

Higher resistance to the disease shown by PHD group fish could be due to improvement of the metabolic activity related to increased liver and plasmatic levels of metabolites such as glycogen, glucose and amino acids as reported for specimens of gilthead seabream (*Sparus aurata*) and *S. senegalensis* farmed under high stocking density and fed a diet supplemented with *S. putrefaciens* Pdp11 [20, 80]. These findings are in agreement with those reporting that specimens of Nile tilapia farmed under crowding stress and fed a probiotic bacterial diet showed higher plasma glucose levels and RNA:DNA ratio than fish fed a conventional diet. These results suggest that probiotic supplemented diets may increase energy availability for metabolic support of stress response, and to show a good tolerance to crowding stress.

Other aspect to be considered is that PHD group specimens have higher number of goblet cells than CHD group fish and in this way they could have higher amount of mucus. In this way, [81] suggested that an enhancement in the number of cells secreting

mucus in specimens of European seabass (*Dicentrarchus labrax*) was related to an improvement in resistance to bacterial infection. The mucus layer overlying the epithelium secreted by goblet cells acts as a medium for protection, lubrication and transport between luminal contents and epithelial cells [82]. The mucus layer provides the first line of defence against microbes and microbial products [83, 84], and in this way cells of pathogenic microorganisms could have more difficulty in passing through the mucus layer and adhering to intestinal cells in fish from PHD groups. However, further experiments to demonstrate this hypothesis are necessary.

The dietary administration of probiotic cells produced an important modulation of the intestinal microbiota of fish and PCA analysis revealed the correlation between the number of total PAS cells and the presence of different clones which only were detected jointly in the intestinal microbiota of fish fed with the probiotic diet and especially in fish from the PHD group at 30 days.

Several studies have correlated the occurrence of increased numbers of goblet cells with the production capacity of eicosanoids in fish [85, 86], and eicosanoids such as prostaglandins (PGs) exert control over inflammation or immunity and PGs receptors are highly expressed on the goblet cells of the intestine [87]. Data concerning the ability of some probiotics to induce changes in the mucus layer causing qualitative alterations in intestinal mucins that inhibit the adherence of enteropathogens have been reported [83, 88]. The transcriptional regulation of mucins has been demonstrated to be mediated by the activation of microorganisms and their products [83, 89, 90]. In relation to these results, the improvement in mucus secretion in fish fed *S. putrefaciens* Pdp11 could be correlated with the intestinal microbiota present in these fish exposed to high stocking densities.

In conclusion, the results derived from this study indicate that *S. putrefaciens* Pdp11 can improve stress tolerance of *S. senegalensis* specimens to high stocking densities by modulating the expression of important immune genes and intestine microbiota and the intestinal condition. Further studies testing the possible mechanisms for this anti-stress role of *S. putrefaciens* Pdp11 and the optimization for its administration (prior and/or during stress situations) will be necessary in order to develop its use in aquaculture.

FIGURE LEGENDS

Supplementary Figure 1. External pathological signs observed in *Solea senegalensis* specimens affected by *Vibrio harveyi* and *V. parahaemolyticus* infection.

Figure 1. Cumulative mortality (expressed as a percentage) detected in the groups of *Solea senegalensis* specimens exposed to high stocking density and fed the normal (CHD 1 and CHD2) and probiotic (PHD1 and PHD2) diets.

Figure 2. Cluster analysis of DGGE patterns of the intestinal microbiota of *Solea senegalensis* specimens fed the control (C) or probiotic (P) diets and exposed to normal (CND and PHD groups) and high stocking densities (CHD and PHD groups) for 19 (Figures 2A and 2C) and 30 days (Figures 2B and 2D).

Figure 3. Non metric multidimensional scaling (NMDS) plots using Bray-Curtis index of DGGE patterns associated with the intestinal microbiota of Senegalese sole

specimens fed control (C) or probiotic (P) diets and exposed to normal (CND and PHD groups) and high stocking densities (the CHD and PHD groups) for 19 (Figures 3A and 3B) and 30 days (Figures 3B and 3D).

Figure 4. Relative abundance of different microbial groups sequenced from the intestinal microbiota of *Solea senegalensis* specimens fed normal or probiotic diets exposed to normal (CND and PND, respectively) or high (CHD and PHD, respectively) densities for 19 and 30 days.

Figure 5. Relative abundance of bacterial species sequenced from the intestinal microbiota of *Solea senegalensis* specimens fed normal or probiotic diets exposed to normal (CND and PND, respectively) or high (CHD and PHD, respectively) densities for 19 and 30 days.

Figure 6. Levels of transcripts of HAMP1, C7, HP y g-LYZ genes. Data are means ± SEM of transcripts molecules/pg of total RNA from at least five fish in each group. Comparisons were made by the Students's-test. Statistical significance is expressed as *** $p<0.001$, ** $p<0.01$ and * $p<0.05$. (***a***) Comparison of specimens exposed to high stocking density *vs* specimens exposed to normal densities; (***b***) Comparison of fish farmed for 30 days *vs* fish farmed for 19 days; (***c***) Comparison of fish treated with the probiotic *vs* fish untreated with the probiotic.

Figure 7. Principal component analysis (PCA) applied to evaluate the relationship between the intestinal microbiota, gene expression, morphometric measurements of the cellular biology of the intestines and mortality rates of *Solea senegalensis* specimens fed normal or probiotic diets exposed to normal (CND and PND, respectively) or high (CHD and PHD, respectively) densities for 30 days.

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Table 1. Species richness (R) and Shannon's index (H') values of the DGGE patterns of intestinal microbiota of Senegalese sole specimens fed control and probiotic supplemented diets and exposed to normal (CND and PND, respectively) or high stocking (CHD and PHD, respectively) densities. a: significant differences ($p < 0.05$) between probiotic diet compared to control diet. b: significant differences ($p < 0.05$) between 19 days compared to 30 days. c: significant differences ($p < 0.05$) between normal density compared to high density.

		R	**H'**
19 days	CND	35 ± 3.27	2.38 ± 0.12
	PND	15 ± 4.28 [a]	1.46 ± 0.24[ab]
	CHD	34 ± 2.03	2.3 ±0.23
	PHD	28 ± 1.29[ac]	2.18 ± 0.28[c]
30 days	CND	37 ± 2.38	2.52 ± 0.85
	PND	18 ± 2.77[a]	1.39 ± 0.20[ab]
	CHD	29 ± 1[bc]	2.38 ± 0.32
	PHD	21 ± 1[ab]	1.62 ± 0.01[abc]

Table 2. Nearest-match identification of 16S rDNA sequences of clone libraries from intestines of Senegalese sole specimens fed control and probiotic supplemented diets and exposed to normal (CND and PND, respectively) or high stocking (CHD and PHD, respectively) densities.

Clone	Closest relative	Similarity (%)	GenBank accession	Presence after 19 days CND	19 days PND	30 days CND	30 days PND	19 days CHD	19 days PHD	30 days CHD	30 days PHD
ϒ-Proteobacteria											
1	*Acinetobacter* sp V(2012)	99	JN820158	-	-	-	-	-	+	-	+
2	*Allivibrio* sp T3	98	KC753328	-	-	-	-	+	-	+	-
3	*Photobacterium damselae* subsp *piscicida* 0029	99	KC466554	-	+	-	-	-	+	-	-
4	*Photobacterium damselae* subsp *piscicida* B04	100	HQ599852	+	+	+	-	-	+	-	-
5	*Photobacterium damselae* subsp *damselae* JCM8968	99	JX916292	-	+	-	-	-	+	-	+
6	*Pseudomonas* sp NJ12	98	KC880363	-	-	-	-	-	+	-	+
7	*Pseudomonas* sp RS-A-12	99	KC430953	-	-	-	-	-	+	-	+

8	*Pseudomonas* sp RS-A-14	100	KC430955	-	-	-	-	+	-	+	-
9	*Pseudomonas* sp s221	99	HM196836	-	-	-	-	-	+	-	+
10	*Pseudomonas putida* PSS5	98	KC426926	-	-	-	-	+	-	+	-
11	*Pseudomonas putida* VSHUB043	98	KC713614	-	-	-	+	-	+	-	+
12	*Pseudomonas putida* ZJB-LLJ	100	JQ824856	-	-	-	-	-	+	-	+
13	Uncultured *Pseudomonas* sp 120516	99	AB773210	-	-	-	-	+	-	+	-
14	Uncultured *Vibrio* sp A3E	100	HQ897301	-	-	-	-	-	+	-	+
15	Uncultured *Vibrio* sp P-F2	99	HQ897566	+	-	+	-	-	-	-	-
16	Uncultured γ-Proteobacteria NL5BD-01-D01	98	FM852401	-	-	-	+	-	+	-	-
17	Uncultured γ-Proteobacteria KK_D06_T-20070605	99	JX435684	+	-	+	-	+	-	-	-
18	*Vibrio* sp CL1	99	JX028553	-	-	-	+	-	+	-	-
19	*Vibrio* sp HE_C5	100	FJ178094	-	+	-	+	-	+	-	+
20	Vibrionaceae P_C5	97	FJ178091	-	-	-	+	+	-	+	+
21	*Vibrio campbelli* VSD714	99	KC534349	-	-	-	-	+	-	+	-

22	*Vibrio ichthyoenteri* FK-1	99	AM181657	-	-	-	-	-	+	-	+
23	*Vibrio ichthyoenteri* SF11070701B	99	JQ904784	-	-	-	-	-	+	-	+
24	*Vibrio natriegens* C170	98	AB719184	+	+	+	+	+	+	+	+
25	*Vibrio parahaemolyticus* CT11	99	JX134442	+	+	+	+	+	+	+	+
26	*Vibrio parahaemolyticus* M2-31	100	KC210810	+	-	+	-	+	-	+	-
27	*Vibrio scopthalmi* D725	99	JF836195	-	-	-	-	-	+	-	+
28	*Vibrio azureus* CD21	99	KC210817	+	-	+	-	-	-	+	-
29	*Vibrio harveyi*	99	HM584117	-	-	-	-	+	-	+	-
β-Proteobacteria											
30	Uncultured β-Proteobacteria T7_182	97	FM206221	+	-	+	+	-	-	-	+
Actinobacteria											
31	*Rhodococcus* sp TM1	99	AY642534	+	-	+	-	-	-	-	-
32	Uncultured Actinobacteria SC3-RK184	98	HF584566	-	-	-	-	+	-	+	-

Firmicutes												
33	Candidatus *Arthromitus*	97	AP122210	+	+	+	-	+	+	-	+
34	Candidatus *Arthromitus* SFB-rat Yit	100	DQ857141	-	+	-	-	-	+	-	+
35	Candidatus *Arthromitus*	99	NR_074450	+	-	+	-	+	-	-	-
36	*Lactobacillus fermentum*	99	AM117157	-	-	-	-	-	+	-	+
37	Uncultured *Lactobacillus* sp	100	AM117177	-	-	-	-	+	-	+	-
Mollicutes												
38	*Mycoplasma* sp C10	99	DQ340196	+	+	+	+	+	+	-	+
39	*Mycoplasma iowae* ATCC 33552	99	JN935877	-	+	-	-	-	-	-	-
40	*Mycoplasma microti* IL371	98	NR_025055	+	+	+	+	+	+	+	-
41	*Mycoplasma muris*	99	HQ897566	+	-	+	-	-	-	+	-
42	Uncultured *Mycoplasma* sp k.b-1	99	HM031446	-	-	-	-	-	+	-	+
Spirochetes												
43	*Brevinema andersonii* MV116	87	L31544	-	-	-	+	+	+	+	+
44	*Brevinema andersonii* ATCC 43811	98	GU993264	-	-	-	-	-	+	-	+
45	Uncultured Spirochete TP-1	100	DQ340184	-	-	-	-	+	-	+	-
46	Uncultured Spirochete 190026	99	AB194657	-	-	-	-	-	`	-	-

Unidentified microorganisms											
47	Uncultured bacterium EXO 15432	97	FM242723	+	+	+	+	+	-	-	+
48	Uncultured bacterium 2B	100	FN396941	-	-	-	-	+	+	+	+

Table 3. SIMPER analysis performed to identify contribution of bacterial groups to total dissimilarity between control and probiotic fed *Solea senegalensis* specimens and exposed to a high stocking density for 19 and 30 days. Values correspond to dissimilarity percentages between the probiotic and control groups

Bacterial group	**Dissimilarity percentages**	
	19 days	**30 days**
γ-Proteobacteria	49.65	23.00
β-Proteobacteria	0.00	9.00
Firmicutes	25.52	7.38
Mollicutes	8.52	10.70
Actinobacteria	8.51	9.00
Spirochetes	1.42	18.00
Unidentified bacteria	6.38	22.92

Table

Table 4. Absolute transcript levels in pooled samples. Quantifications were carried out in total RNA from pooled livers (≥ 5 individuals/condition). Data are transcript molecules per pg of total RNA. Numbers in boldface indicate ≥2 fold up-regulations while numbers in italic indicate ≥2 down-regulations relative to the respective controls. [a] Comparison between specimens exposed to high stocking densities vs specimens exposed to normal stocking densities; [b] Comparison between specimens farmed at 30 days vs specimens farmed at 19 days; [c] Comparison between specimens fed the probiotic diet vs specimens fed normal diet. Gene symbols are according to the NCBI Gene database.

	19 days				**30 days**			
Transcript	**CND**	**PND**	**CHD**	**PHD**	**CND**	**PND**	**CHD**	**PHD**
C3	8182	7124	6203	5308	4622	5128	6311	5308
C7	120	143	*56*[a]	*63*[a]	81	104	**665**[a,b]	*118*[c]
HAMP1	4.10	3.50	2.80	2.70	3.00	2.00	**239**[a,b]	*3.60*[c]
HP	280	202	*101*[a]	*85*[a]	148	155	**819**[a,b]	**202**[b,c]
HSP70	0.08	**0.17**[c]	*0.04*[a]	*0.02*[a,c]	**0.67**[b]	*0.05*[bc]	*0.17*[ab]	*0.03*[c]
HSP90AB	224	257	382	311	172	224	248	424
g-LYZ	2.30	2.80	*1.30*[a]	*0.85*[a]	2.60	2.70	*0.99*[a]	*1.0*[a]
NARS	6.30	5.20	*3.10*[a]	*2.30*[a]	3.20	2.90	**20**[a,b]	*3.90*[c]
NCCRP1	0.17	0.12	0.10	0.08	0.11	0.16	**7.40**[a,b]	*0.10*[c]
TFNAIP9	24	38	47	44	23	37	**863**[a,b]	*41*[c]

Table

Table 5. Representation of the average villus height and diameter and number of goblet cells in the gut of *Solea senegalensis* specimens. Data are represented as mean ± standard error. Different letters denote in the same column denote significant differences.

				Goblet Cells		
Day	**Treatment**	**VH (µm)**	**D (µm)**	**PAS+**	**PAS+AB+**	**Total**
19	CND	574.4±16.4^{a}	2572.3±34.1^{a}	56.0±13.1^{a}	42.3±8.2^{a}	98.4±17.4^{a}
	PND	595.3±15.8^{a}	2384.0±54.8^{b}	70.3±26.5^{a}	44.5±17.7^{a}	114.8±34.3^{a}
	CHD	521.7±10.6^{b}	1750.8±27.8^{c}	84.9±19.9^{a}	59.5±17.2^{a}	144.4±33.8^{a}
	PHD	463.1±9.9^{c}	1680.6±30.9^{cd}	40.0±10.3^{a}	34.8±6.4^{a}	94.8±13.5^{a}
30	CND	639.7±11.6^{a}	2187.3±27.9^{a}	36.5±9.5^{a}	33.1±11.5^{a}	69.6±15.6^{a}
	PND	509.9±9.6^{b}	1749.6±19.5^{b}	31.0±5.6^{a}	3.5±1.4^{b}	34.5±6.1^{a}
	CHD	433.7±9.1^{c}	1775.4±26.3^{b}	44.9±18.4^{a}	39.7±17.2^{ab}	84.6±35.1^{b}
	PHD	480.0±9.1^{b}	1708.4±37.0^{b}	83.1±12.5^{b}	38.5±8.6^{c}	121.6±17.1^{c}

CND: control group farmed under a normal density; PND: Probiotic group farmed under a normal density; CHD: control group farmed under a high density; PHD: Probiotic group farmed under a high density. VH: villus height in the gut of *Solea senegalensis*; D: diameter of gut of *Solea senegalensis*; PAS+: Number of goblet cells stained with periodic acid technique in $1000\mu^2$; PAS+AB+: Number of goblet cells stained with periodic acid and alcian blue techniques in 1000 μ^2. Different letters in the same column denote significant ($p < 0.05$) differences.

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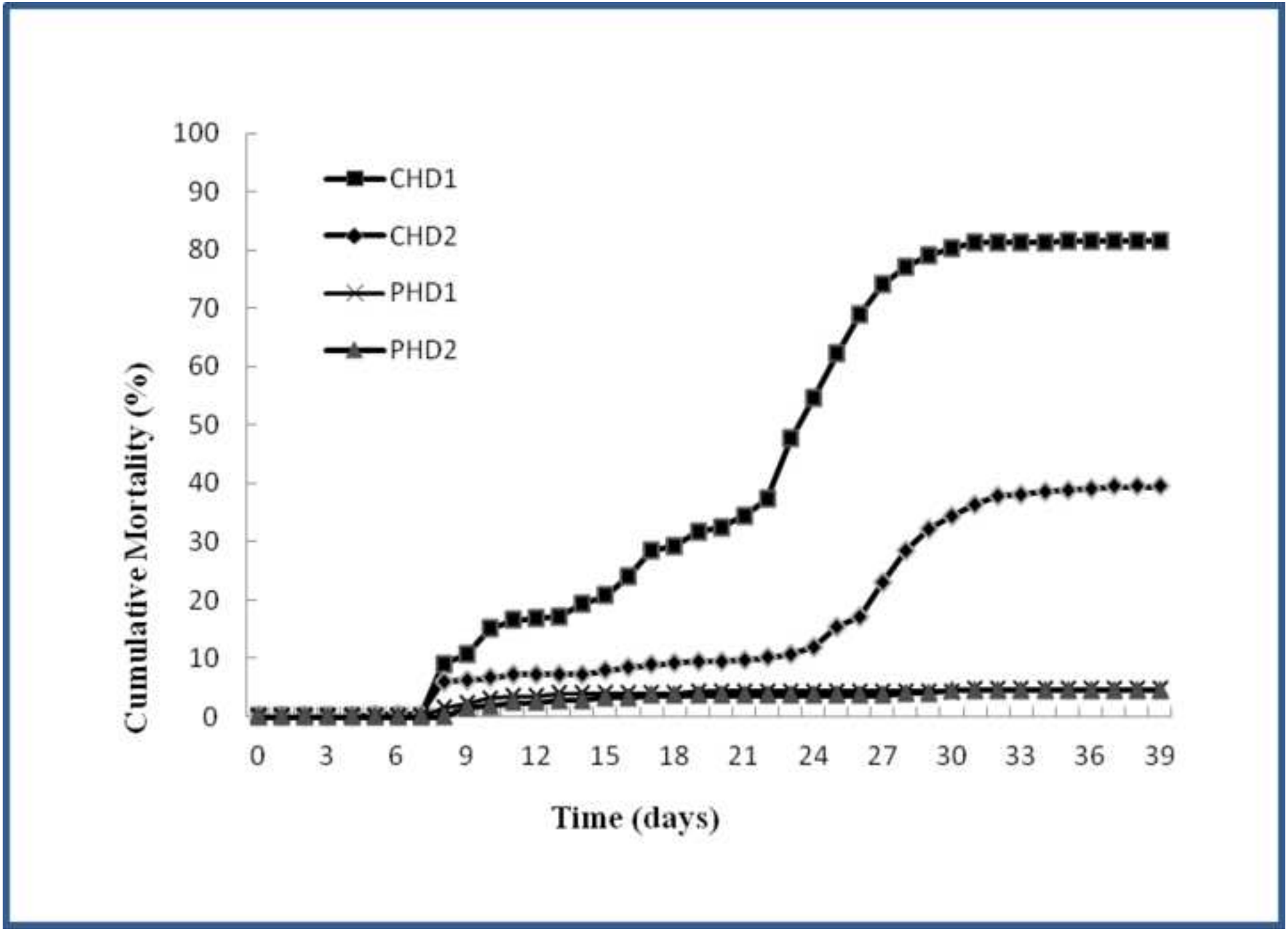

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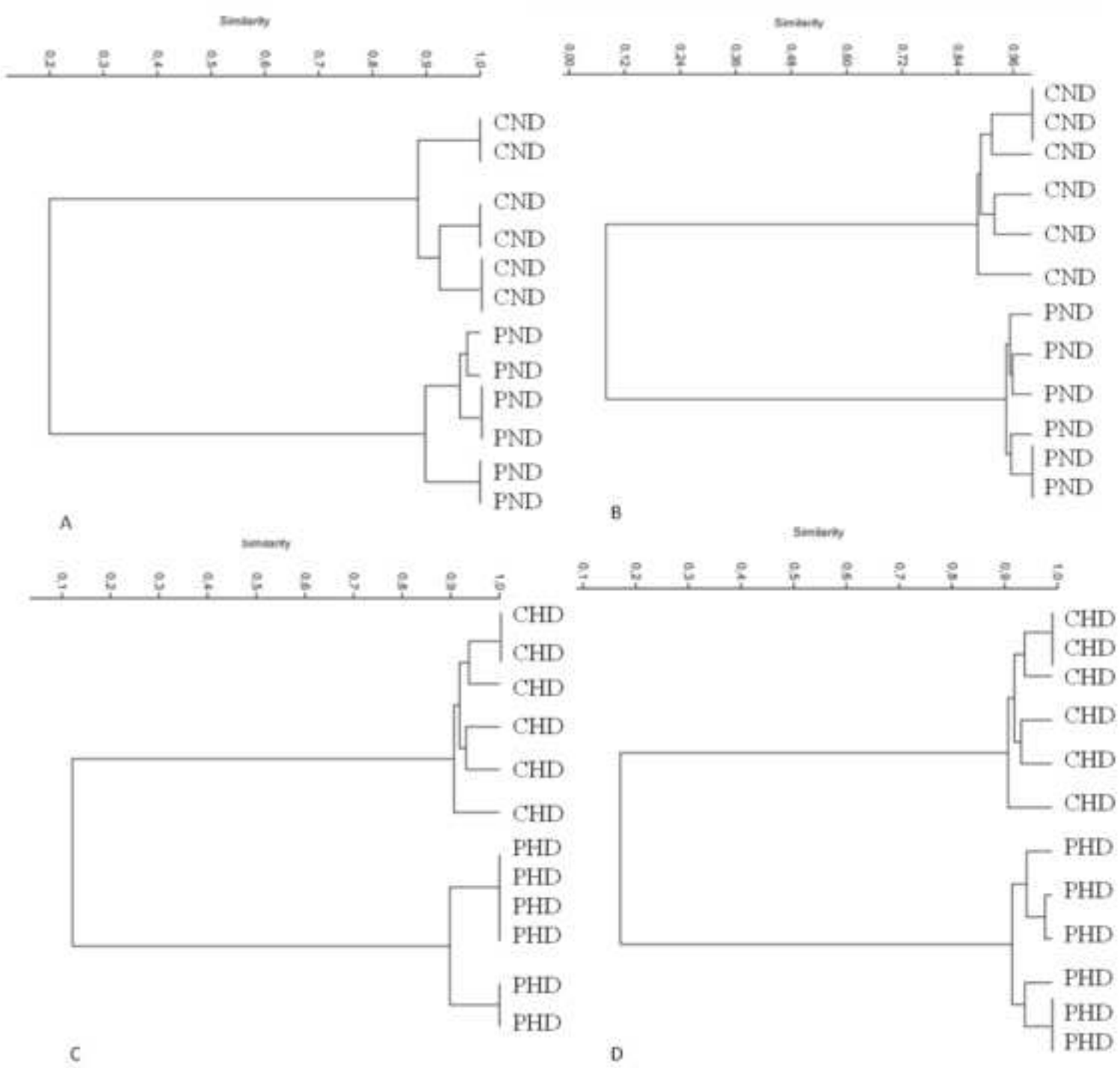

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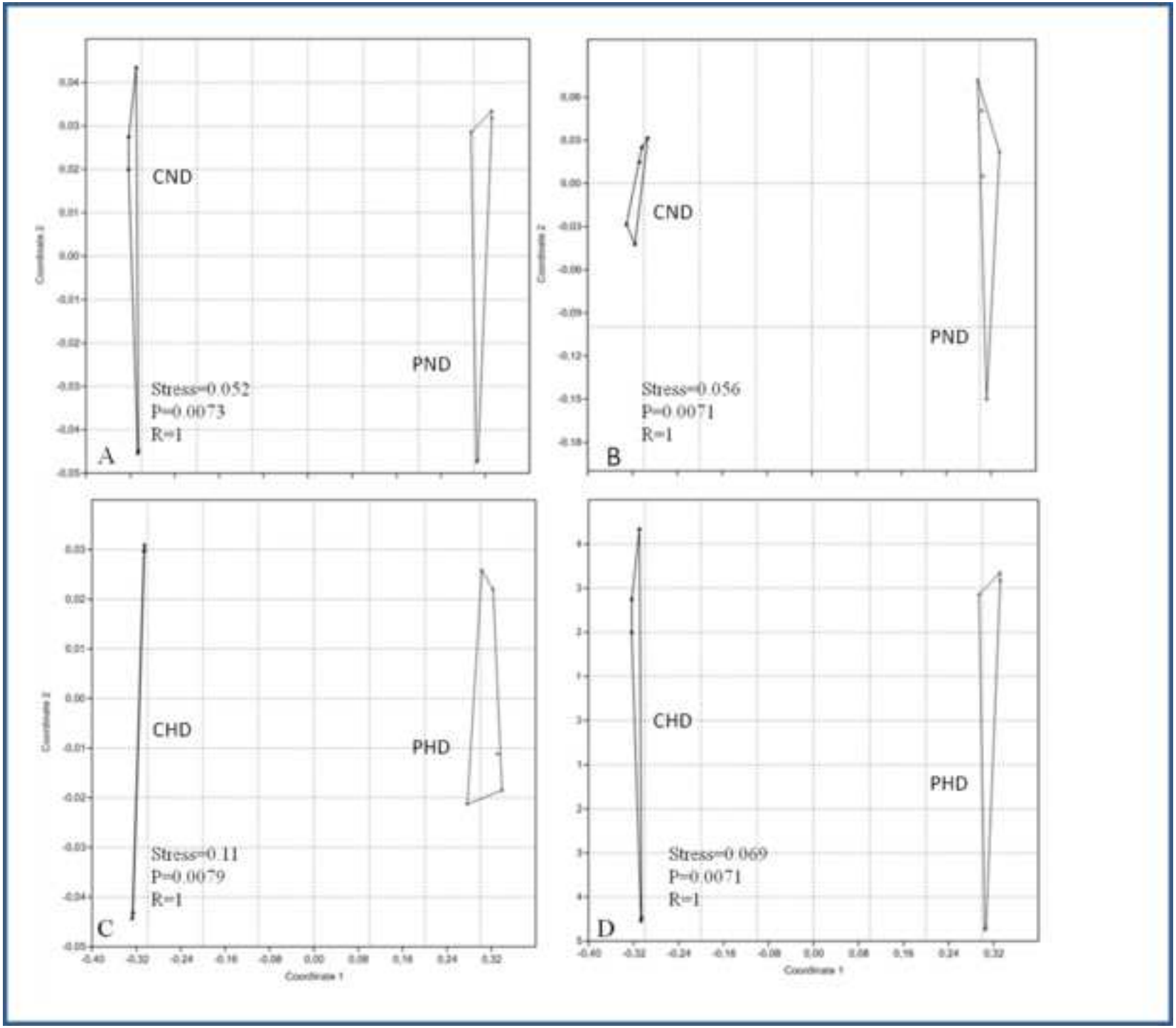

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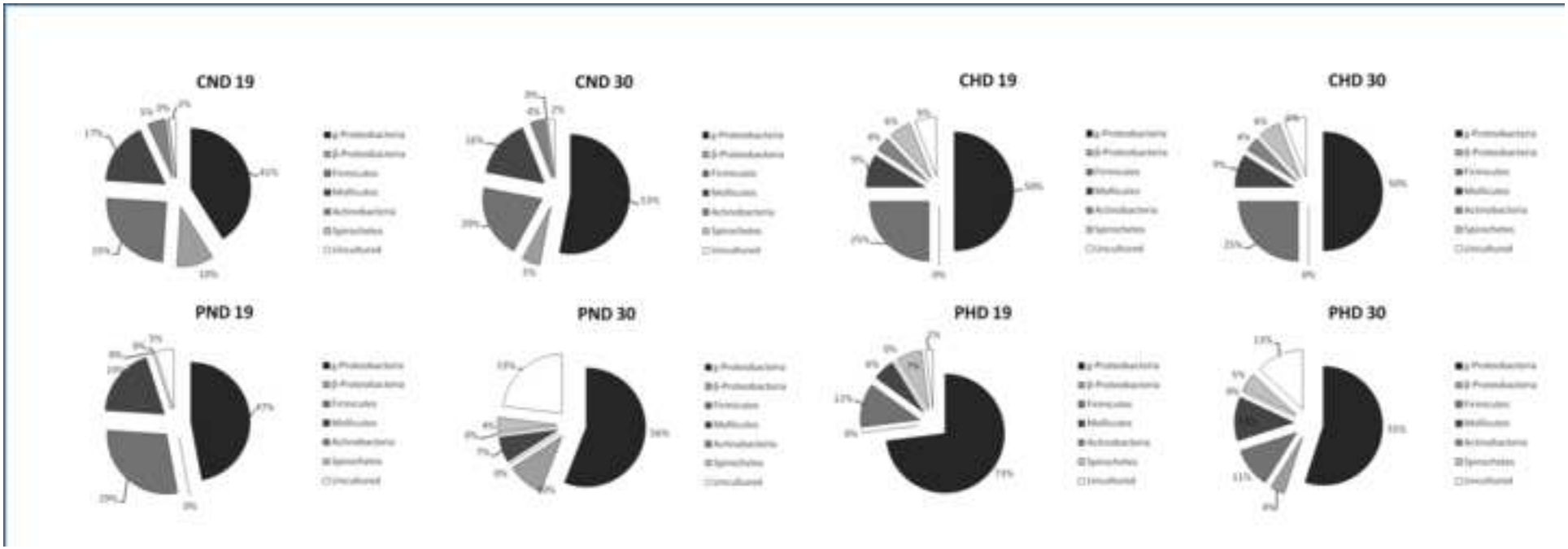

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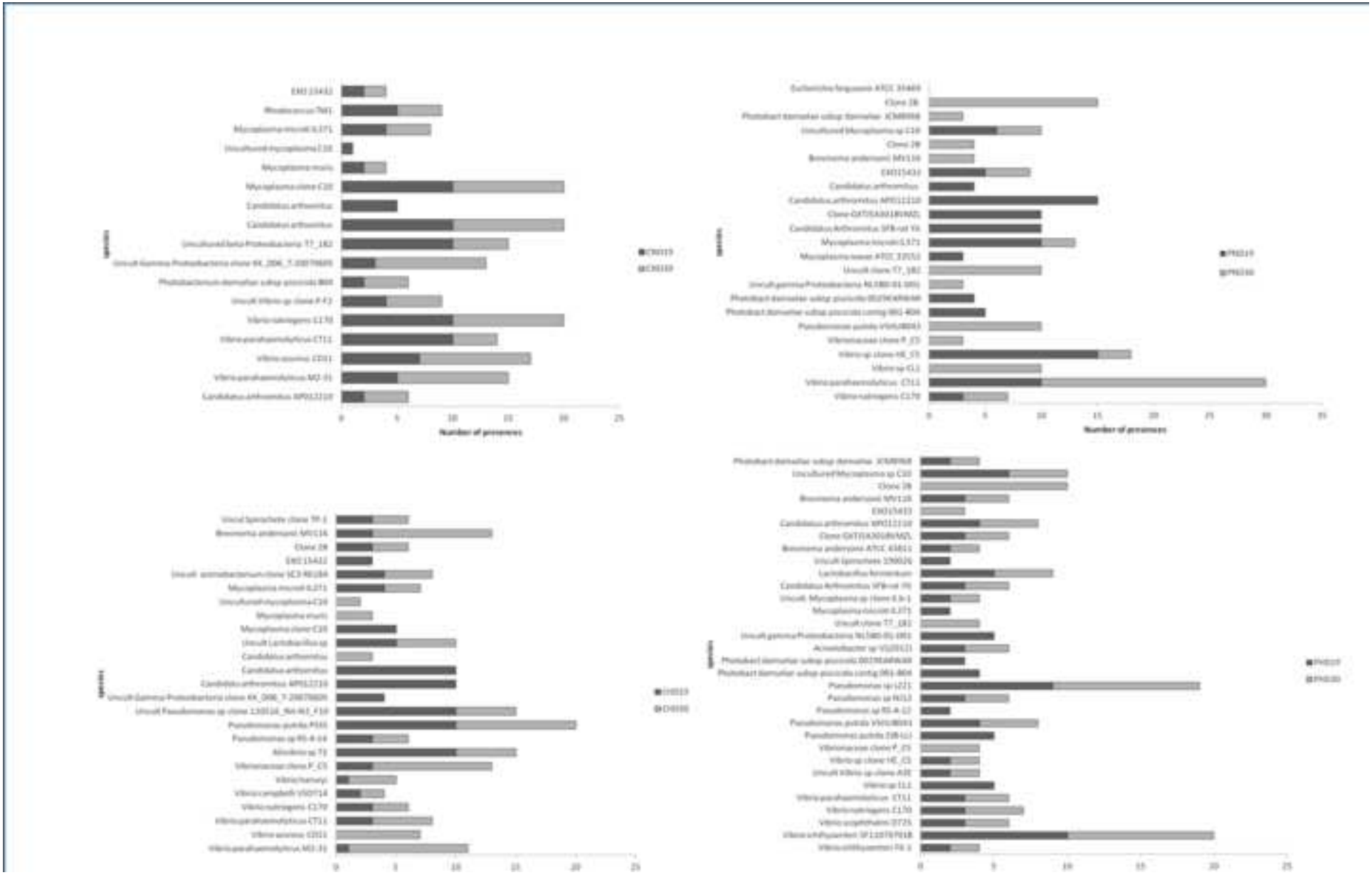

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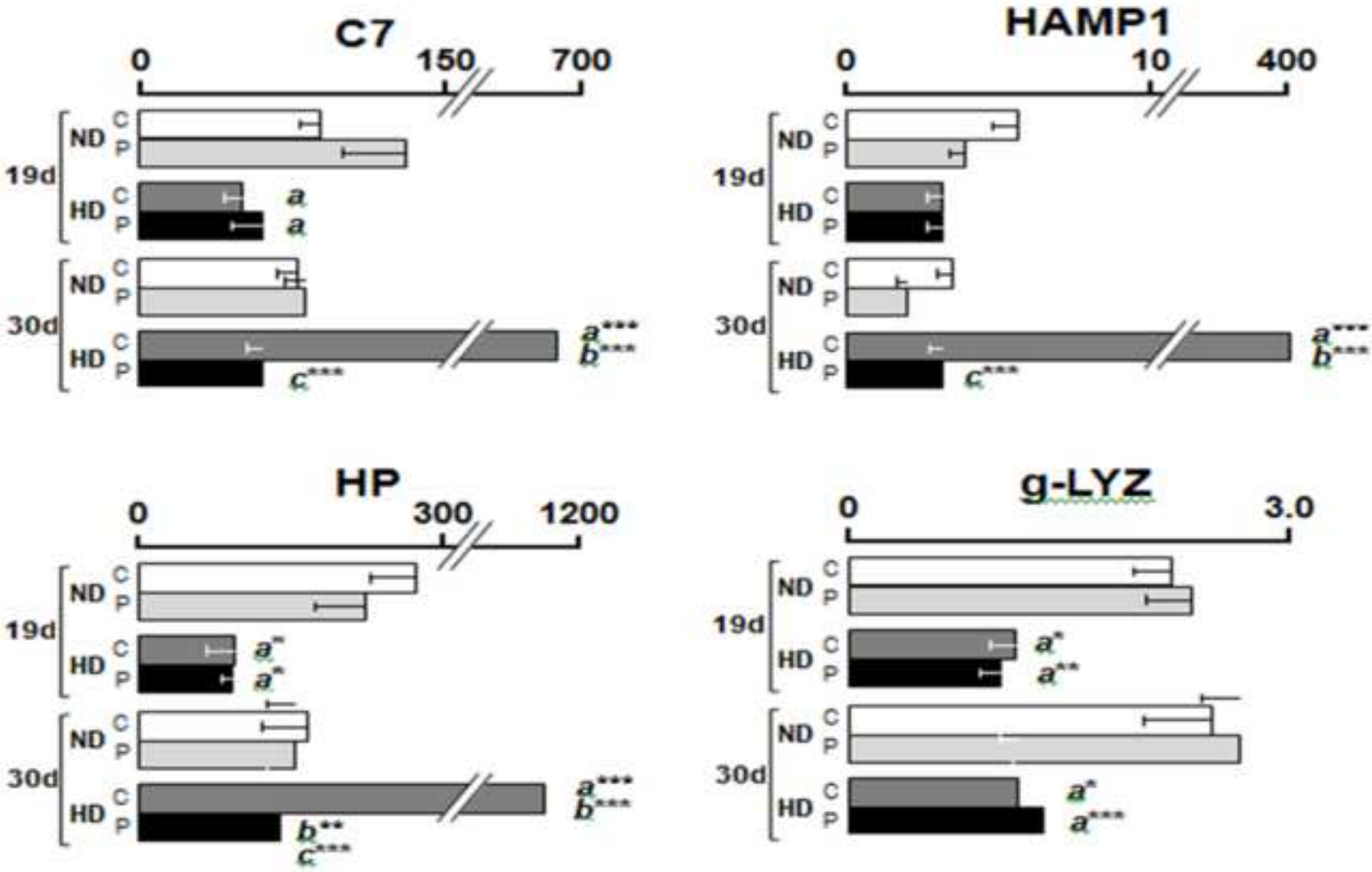

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